



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ :		(11) International Publication Number:	WO 98/13378
C07K 14/35, A61K 39/04	A1	(43) International Publication Date:	2 April 1998 (02.04.98)
(21) International Application Number:	PCT/NL97/00536	(81) Designated States:	AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).
(22) International Filing Date:	25 September 1997 (25.09.97)	(30) Priority Data:	
96202701.7	26 September 1996 (26.09.96) EP	(34) Countries for which the regional or international application was filed:	NL et al.
(71) Applicant (for all designated States except US): RIJKSUNIVERSITEIT TE LEIDEN [NL/NL]; Stationsweg 46, NL-2312 AV Leiden (NL).		(72) Inventors; and	
(75) Inventors/Applicants (for US only): KONING, Frits [NL/NL]; Zadelmaker 20, NL-2353 WR Leiderdorp (NL). DRIJFHOUT, Jan, Wouter [NL/NL]; Da Costastraat 41, NL-2321 AK Leiden (NL).		(74) Agent:	SMULDERS, Th., A., H., J.; Vereenigde Octroibureaux, Nieuwe Parklaan 97, NL-2587 BN The Hague (NL).

(54) Title: MANNOSYLATED PEPTIDES

(57) Abstract

T cells mediate specific immune reactions. They recognize (foreign) protein fragments – peptides – that are bound in the peptide binding groove of MHC molecules. These peptide MHC complexes are generated intracellularly and subsequently transported to the cell surface for recognition by T cells. A rate limiting step in the MHC restricted presentation of (foreign) peptides derived from extracellular sources is therefore the uptake of such antigens by antigen presenting cells and the delivery to the intracellular compartment(s) where the MHC-peptide complexes are formed. Professional antigen presenting cells like dendritic cells and macrophages take up antigens by macropinocytosis and mannose receptor mediated endocytosis. We have explored the possibility that the mannosylation of otherwise non-mannosylated antigens would lead to enhanced uptake and MHC restricted presentation of (fragments of) such antigens by mannose receptor positive cells. Using mannose receptor positive cultured human dendritic cells as antigen presenting cells we found that the mannosylation of antigenic peptides resulted in a 300 – 10.000 fold enhanced potency to stimulate MHC class II restricted peptide-specific T cell clones compared to non-mannosylated peptides. These results indicate that mannosylation of antigens leads to selective targeting and subsequent superior presentation by dendritic cells, a result which may be applicable in for example vaccine design.

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		

Title: Mannosylated Peptides

The present invention relates to the field of immunology, in particular cellular immunology. More specifically it relates to the presentation of antigen-derived peptides on the surface of antigen presenting cells (APC's) in the context of an MHC-molecule.

T cells play a central role in the immune system. They mediate specific immune reactions against pathogens and tumors but also cause autoimmune diseases and are responsible for the initiation of transplant rejection. Research in the last ten years has revealed that T cells recognize protein fragments - peptides - that are bound in the peptide binding groove of MHC-molecules. These peptide MHC-complexes are generated intracellularly and subsequently transported to the cell surface in order to allow detection by T cells. Peptides derived from intracellularly synthetized proteins most commonly are found in association with MHC-class I molecules. Peptides derived from endocytosed proteins synthetized outside the cells are most commonly found in association with MHC-class II molecules. It is well established that primary T cell responses are initiated most efficiently if peptides are presented by MHC-molecules on a specialized class of antigen presenting cells: cells of the dendritic lineage. Dendritic cells are present in low numbers in peripheral blood, in lymphoid tissues and epithelia. Their extraordinary capacity to induce primary immune responses is attributed to the high cell surface expression of MHC-class I and class II molecules, adhesion molecules and high co-stimulatory activity. Other, currently unknown proteins may also contribute to this process.

One of the problems encountered in trying to induce T cell responses to proteinaceous antigens is that peptides are not efficiently presented by APC's. This problem may lie in the uptake of the proteinaceous antigen or a peptide

derived therefrom by the APC, in the processing of the antigen (cleavage sites and/or transport signals for transport to the right compartment and eventually the surface of the APC, in the complexing of the peptide with the MHC-molecule, etc). Any of these problems may be the reason why peptide or protein based vaccines for many specific pathogens have met with limited success. The present invention identifies an important limiting step in antigen processing. Said limiting step is the uptake of proteinaceous antigens or peptides derived therefrom.

The present invention improves the uptake of said proteinaceous antigens or peptides by APC's by providing said antigen or peptide with one or more carbohydrate moieties which has an affinity for a mannose receptor on the surface of an APC.

Recent reports have revealed that both cultured monocyte-derived human dendritic cells and murine dendritic cells in situ express mannose receptors and that this mannose receptor is used for the efficient endocytosis of mannosylated proteins.

It has been shown that DCs play a critical role in antigen presentation in vivo and that they can prime virgin T cells (R.M. Steinman, Annu. Rev. Immunol. 9, 271-296 (1991)). It has also been shown that receptor-mediated uptake of antigens can effect antigen presentation via MHC class II (A. Lanzavecchia, Annu. Rev. Immunol. 8, 773-793 (1990)). Recently, F. Sallusto et al. (J. Ex. Med. 182, 389-400 (1995)) have shown that endocytosis of antigens by DCs occurs in various ways including fluid phase uptake, macropinocytosis and MR-mediated uptake, and that macromolecules that are endocytosed by one of the latter two ways of receptor-mediated endocytosis are concentrated in the MHC class II compartment. R.M. Steinman and J. Swanson (J. Exp. Med. 182, 283-288 (1995)) raised the question whether DCs might have receptors that mediate absorptive uptake and deliver these as peptides to MHC products. However, no

experimental data have been presented in this publication to verify the hypothesis. A. Lanzavecchia (Curr. Opin Immunol. 8, 348-354 (1996)) has reported that DCs can present low concentrations of soluble antigen by endocytosis via 5 macropinocytosis and that the complexing of antigens with antibodies and the mannosylation of antigens can be employed to target antigens or toxins to dendritic cells. No mention is made of the possibility of enhancing antigen presentation on dendritic cells by targeting the mannose receptor. The 10 statements are based on personal communications and no experimental data, like the nature of the antigens and the way of mannosylation are presented. European Patent Application 0 659 768 A2 (publication date 28.06.1995) describes various in vivo experiments with conjugates of 15 peptides/proteins with a carbohydrate polymer (especially mannan). Immune responses could be measured following treatment with a conjugate of a fusion protein with a carbohydrate polymer in mice. No data were presented on the effectiveness of protein-carbohydrate monomer conjugates nor 20 on that of conjugates of peptides with carbohydrate monomers or polymers. There is no disclosure on the mannose receptor or its use for enhancing antigen presentation.

Thus the invention provides a method for enhancing uptake of a proteinaceous antigen or a peptide derived 25 therefrom by antigen presenting cells having a mannose or similar receptor, whereby the antigen is provided with at least one and preferably at least two mannose groups or a functional equivalent thereof. In the context of this application it is clear that (partial) agonistic peptides and 30 antagonistic peptides are useful (as also explained in the experimental part) to be presented to T cells. In the one case the response can be enhanced, in the other case blocked. Both kinds of peptides may be mannosylated themselves or may 35 be part of larger mannosylated structures, such as proteinaceous "antigens".

An important aspect of the present invention is that the antigen or peptide is targeted to a mannose receptor present on APC's such as the mannose receptor present on dendritic cells. For this purpose a mannose group (or more than one) attached to the antigen or the peptide is of course very suitable, but other glycosyl groups may also be used as long as they have a significant binding affinity for a mannose receptor. The structure of the total glycosyl group is not overall important, as long as at least one carbohydrate moiety is recognized by the mannose receptor. The number of glycosylic residues may thus also vary. Our experiments show that the number of carbohydrates attached to the peptide ($n=1-6$ or preferably $2-6$) only marginally influences the presentation of the peptide on the cell surface. It is clear that the use of a different number of carbohydrate moieties on the peptide ($n>6$) than the number we used will show a comparable effect. Other moieties that have binding affinity for mannose receptors on APC's include, but are not limited to b-L-Fucose, b-D-Glucopyranose, b-D-N-Acetylgalactosamine, etc. This holds true also for modified carbohydrates which have affinity for the mannose receptor like carbohydrate analogs possessing instead of an oxygen atom, an anomeric carbon or an anomeric sulfur atom. It could be a derivative containing one or more halogen atoms replacing one or more hydroxyl(s). It may be modified carbohydrate derivatives with seven, eighth or more atom rings.

The improvement in the uptake by targeting a mannose receptor and the transport by said mannose receptor to the right compartment in the cell for processing, and/or complexing to an MHC-I or MHC-II molecule results in a very efficient presentation of the mannosylated peptide on the surface of the APC's, in particular those of dendritic origin.

Our results demonstrate that mannosylation of peptides leads to very efficient uptake and presentation of these peptides by dendritic cells at low concentrations of peptide. Depending on the T cell clone used, 300 - 10,000 fold less

mannosylated peptide is required for the induction of T cell proliferation when compared with non-mannosylated peptides. Furthermore, only a short exposure to mannosylated peptide is required for the generation of sufficient MHC-peptide complexes to allow the induction of T cell proliferation.

5 Similarly we demonstrate that 1,000 fold less of a mannosylated peptide with antagonistic properties is required for the induction of T cell non-responsiveness when compared to non-mannosylated peptide. This indicates that the mannose receptor expressed on the cell surface of cultured dendritic cells mediates very efficient transport of mannosylated peptides to the intracellular compartment where (newly synthetized) MHC-class II molecules bind peptides. We also observed that mannosylated peptides are presented more

10 efficiently than non-mannosylated peptides when human peripheral blood mononuclear cells are used as antigen presenting cells. This shows that enough mannose receptor positive antigen presenting cells are present in these peripheral blood preparations to allow enhanced presentation.

15 20 It is known that in peripheral blood approximately 1% of the cells are immature dendritic cells that could mediate this effect.

In the peptides we used, the carbohydrate moieties were attached to the N-terminus of the peptides. It is clear that the same biological effect would be obtained with peptides in which the carbohydrate moieties are attached to the C-terminus, or to parts of the peptides between the N-terminus and the C-terminus e.g. to side chains. Also peptides having carbohydrate moieties attached to combinations of the possibilities mentioned above are

25 30 expected to have the same biological effect.

It is clear that peptide-like compounds (peptoids and/or peptidomimetics) to which carbohydrates are attached can have the same effect. Peptoids could be peptide-like compounds having a modified backbone structure because they contain unnatural amino acids, D-amino acids, amino acid like building blocks such as β α -amino acids, amide bond

mimics like reduced peptide bonds, sulfonamide bonds, sulfinamide bonds, ester bonds, ether bonds, disulfide bonds, thioether bonds or saturated an unsaturated carbon-carbon bonds. Peptoids could be PNA also.

5 It is unlikely that the way the glycosylic groups are attached to the peptides is of crucial importance for the biological effect. Other ways of attaching the glycosylic group(s) could be the reaction of other amine reactive groups in the glycosylic moiety like active esters, acid chlorides, 10 acid bromides, acid iodides, symmetric anhydrides, unsymmetric anhydrides, in situ preactivated carboxylic acids to amines in the peptides. It could be the reaction of activated carboxylic groups in the peptide to an amine in the glycosylic moiety. It could be the reaction of a thiogroup in 15 the peptide to a thioreactive group in the carbohydrate moiety like an S-Npys, an S-Nps, a thiol, an acetyl bromide, an acetyl iodide. It could be the reaction between a thiogroup in the carbohydrate to a thiolreactive group in the peptide, like an acetyl bromide, an acetyl iodide. It could be an ester forming reaction between a activated carboxylic acid, an acid 20 chloride, an acid bromide, an acid iodide, an in situ activated carboxylic acid in the carbohydrate moiety and a hydroxy group in the peptide. It could be any nucleophilic substitution reaction between a modified carbohydrate and a 25 suitable reactive center in the peptide. It could be any addition reaction of a modified carbohydrate and a suitable reactive center in the peptide.

It is evident that this type of compound could be synthesized completely by solid phase strategies. Such 30 compounds could be synthesized by performing peptide synthesis using amino acids bearing carbohydrate moieties in the side chain. These glycopeptides could be made by using amino acids functionalized on the a-amino group with carbohydrate moieties that are introduced at the N-terminus 35 of the peptide to a branched cluster of amino acids possessing various amino functionalities.

Multiple glycosylic moieties in the peptide could also be attached to the peptide by a dendrimer type of structure. These compounds can be synthesized by using bis-amino acids (like lysine, ornithine and diaminopropionic acid). During synthesis the protecting groups are removed from both amino groups before coupling of the next amino acid. This procedure yields constructs with a branched structure, with the possibility to couple glycosylic moieties to the branches.

The best results will of course be obtained if the peptides which are provided to the mannose receptor or which are the result of processing of the proteinaceous antigen delivered to the mannose receptor also have the other characteristics favorable to APC presentation in the context of MHC-class I or MHC-class II molecules. These include, the right size to fit on the MHC-molecule, the right anchor residues to fit in the respective MHC-molecules, transport signals for arriving at the MHC-molecule and for the proteinaceous antigen the proper cleavage sites for the processing enzymes of the APC's.

The mannosylated proteins and/or peptides, possibly in combination with mannose receptor expressing dendritic cells or dendritic-like cells, can be used for a number of purposes. These include, but are not limited to:

- The use of mannosylated proteins and/or peptides for vaccination purposes. Amongst others this includes vaccination protocols for the induction of immunity against pathogens like (myco)bacteria, viruses, yeasts, fungi, helminths and parasites but also vaccination protocols designed to enhance or elicit tumor specific responses.
- For instance desired mannosylated (peptide) antigens may comprise (mixtures or combinations of) known antigenic proteins or peptides, or one or more covalently linked known T helper and cytotoxic T cell epitopes. For example, a known T helper cell epitope that evokes a memory response and thus may aid in eliciting a CD8 response to the

cytotoxic T cell epitope, for example a tumor specific peptide.

Another delivery system may utilize mannosylated liposomes or vesicles into which the desired antigenic proteins or 5 peptides have been incorporated.

- T cells reactive with autoantigens have been described and are thought to play a role in the development of autoimmune diseases like type I diabetes and reumathoid arthritis.

Similarly, alloreactive T cells, responsible for graft 10 rejection after organ transplantation and the development of graft versus host disease after bone marrow transplantation, have been shown to be often specific for or dependent on the presence of particular MHC bound peptides. Immune intervention can be envisioned at the level of competition 15 for binding to MHC molecules. For this purpose synthetic compounds with high affinity for the peptide binding groove must be designed that are able to compete for binding to MHC molecules and thus block the binding of immunogenic autoantigen derived peptides to these MHC molecules. This 20 approach is hampered by the relative inefficient uptake of peptides by antigen presenting cells and can be facilitated by the use of mannosylated peptides.

- The use of mannosylated peptides with antagonistic 25 properties for the induction of T cell non-responsiveness. Peptides with antagonistic properties have been described. These peptides differ from the agonist in the nature of the amino acid(s) at T cell receptor contact points and can 30 (partially) block the response to the agonist. The in vivo application of peptides with antagonistic properties may be hampered by the excess of antagonist peptide that is usually required to achieve efficient blocking of the T cell response and the short half-life of peptides in vivo. The use of mannosylated peptides with antagonist properties may be 35 applicable for the efficient targetting of the peptides to

dendritic cells and other mannose receptor positive cells and thus escape from degradation and more efficient loading on MHC molecules, leading to the induction of T cell non-responsiveness at much lower peptide concentrations.

- 5 Vaccines may of course contain usual additives and/or adjuvantia and/or carriers as may other pharmaceutical preparations.

The invention will be explained in more detail in the following experimental part.

10 **EXPERIMENTAL PART**

Introduction

In our experiments we used various mono- and multiple-mannosylated peptides in which the mannose groups had been attached to the N-terminus of the peptides. For this we synthesized peptides containing in addition an N-terminal lysine and peptides containing in addition various N-terminal lysines (n=1-5).

Mannosylation was performed by reaction of the N-terminal amine group and the amine group(s) in the side chains of the lysine(s) with an isothiocyanate derivative of mannose, yielding an thioureum linkage between the peptide and the mannose moieties.

Herein we use the word mannose frequently. In the experiments the word mannose should be read as mannose, everywhere else the word mannose should be read as "every carbohydrate moiety that shows affinity for the mannose receptor".

The formation and expression of antigenic MHC-peptide complexes was measured using pro

30 proliferative peptide specific T cell clones. The clones were cultured together with HLA-DR matched cultured dendritic cells in the presence or absence of a concentration range of the appropriate peptide. The peptides were either non-mannosylated or mannosylated. Activation of the T cell clones

was measured by the incorporation of [³H]-thymidine after 48 hours of culture. In these experiments we observed that 300- to 10.000 fold less of the mannosylated peptide was required for the induction of half-maximal proliferation compared to 5 the non-mannosylated peptide.

MATERIALS AND METHODS

Synthetic peptides

Peptides were synthesized by solid phase strategies on an automated multiple peptide synthesizer (Abimed AMS 422) 10 (Gausepohl et al., 1990^a). Tentagel S AC (Rapp, Tübingen, Germany), (Sheppard et al., 1982; Rapp et al., 1990), a graft polymer of polyethyleneglycol and polystyrene to which the C-terminal amino acid of the peptide had been attached by the manufacturer, was used as a resin (loading 0.2 μ eq, 15 particle size 90 μ m). Repetitive couplings were performed by addition of a mixture of 90 μ l 0.67 M PyBOP (Gausepohl et al., 1990^b; Coste et al., 1990) in NMP, 20 μ l NMM in NMP 2/1 (v/v) and 100 μ l of a 0.60 M solution of the appropriate Fmoc 20 amino acid (Fields et al., 1990) in NMP (6-fold excess) to each reaction vessel. At 70% of the reaction time approximately 50 μ l dichloromethane were added to each reaction vessel. Fmoc-deprotection was performed by adding 3 times 0.8 ml of piperidine/DMA 1/4 (v/v) to each reaction vessel. Coupling- and deprotection times were increased as 25 the synthesis proceeded, starting with 30 min and 3 times 3 min, respectively. Washings after coupling and Fmoc- deprotection steps were done with 6 times 1.2 ml DMA. After synthesis the peptidylresins were washed extensively with DMA, dichloromethane, dichloromethane/ether 1/1 (v/v) and ether respectively, and dried in the air (at least 2 h). 30 Cleavage of the peptide and removal of the side chain protecting groups was performed by adding 6 times 200 μ l TFA/water 19/1 (v/v) at 5 min intervals to each reaction vessel. 2.5 h after the first TFA addition the peptides were

precipitated from the combined filtrates by the addition of 10 ml ether/pentane 1/1 (v/v) and cooling to -20°C. The peptides were isolated by centrifugation (-20°C, 2,500g, 10 min). After titration of the pellet with 10 ml ether/pentane 1/1 (v/v) and isolation by the same procedure, the peptides were dried at 40°C for 15 min. Each of the peptides was dissolved in 2 ml water (or 2 ml 10 vol% acetic acid), the solution frozen in liquid nitrogen for 3 min, and lyophilized while being centrifuged (1,300 rpm, 8-16 h). The peptides were stored at -20°C until use. The purity of the peptides was determined by analytical reversed phase HPLC using a water-acetonitrile gradient containing 0.1 % TFA, and proved to be at least 70% pure (UV 214 nm). The integrity of the peptides was determined by laser desorption time-of-flight mass spectrometry (MALDI-TOF MS) on a Lasermat mass spectrometer (Finnigan MAT, UK). About 5 pmol of the peptide in 0.5 µl water/acetonitrile containing 0.1% TFA was mixed with 0.5 µl of matrix solution (ACH, 10 mg/ml in acetonitrile/water 60/40 (v/v) containing 0.1% TFA) and applied to the instrument. Calibration was performed with peptides of known molecular mass (1422.7 or 1896.1), either as external or as internal references.

Attachment of the carbohydrate moieties to the peptides

In the procedure described below α-D-mannopyranosyl-phenylisothiocyanate was used for the introduction of the mannose moieties in the peptides. The same procedure was followed for the synthesis of the peptides containing other glyco moieties.

To a solution of 2 mg of a synthetic peptide in 200 µl DMSO was added 2 mg of α-D-mannopyranosylphenylisothiocyanate (Cas 96345-79-8) per amino group in the peptide and 1 µl of NMM per amino group in the peptide, respectively.

The reactionmixture was stirred overnight, afterwhich the excess α-D-mannopyranosylphenylisothiocyanate was

hydrolysed in 1 h by the addition of 25 μ l Tris.HCL (pH 9.5, 1 M) per mg a-D-mannopyranosylphenylisothiocyanate used.

The mannosylated peptides were purified by gradient elution on a Jasco HPLC system, containing a Jasco PU-980 intelligent HPLC Pump, a Jasco LG-980-02 Ternary Gradient Unit, a Jasco UV-975 intelligent UV/VIS Detector, a Jasco AS-950 intelligent Sampler, controlled by a JCN Cobra Excel 486 PC using Borwin vs 1.20 for Windows (B&L Systems, Maarssen, The Netherlands) equipped with a RP-HPLC Vydac 218TP1010 column (1.0 x 25cm) using a flow of 5 ml/min and a linear gradient of water/acetonitrile/TFA 949/50/1 to water/acetonitrile/TFA 249/750/1 in 28 min. Detection was at 225 nm. The purified peptides were lyophilized in aliquots and stored at -20 °C until use.

The purity of the mannosylated peptides was checked on a Waters LCM-1 system using Millenium Chromatography Manager (Waters) and equipped with a RP-HPLC Nucleosil 100-5C18AB column (0.4 x 25 cm) (Macherey-Nagel, Düren, Germany), using a flow of 1 ml/min and a linear gradient of water/acetonitrile/TFA 949/50/1 to water/acetonitrile/TFA 249/750/1 in 23 min. Detection was at 214 nm.

All mannosylated peptides were analysed by MALDI-TOF MS on a Lasermat mass spectrometer (Finnigan MAT, UK). About 5 pmol of the peptide in 0.5 μ l water/acetonitrile containing 0.1% TFA was mixed on a sample slide with 0.5 μ l of matrix solution (ACH, 10 mg/ml in acetonitrile/water 60/40 (v/v) containing 0.1% TFA). The sample was allowed to dry in the air for about 10 min and applied to the instrument. Calibration was performed with peptides of known molecular mass (1422.7 or 1896.1), either as external or as internal references.

The following peptides were synthesized:

K1P1	K-T-I-A-Y-D-E-E-A-R-R-G
K2P1	K-K-T-I-A-Y-D-E-E-A-R-R-G
K3P1	K-K-K-T-I-A-Y-D-E-E-A-R-R-G
K4P1	K-K-K-K-T-I-A-Y-D-E-E-A-R-R-G
K5P1	K-K-K-K-T-I-A-Y-D-E-E-A-R-R-G
K1P1.1	K-T-I-Q-Y-D-E-E-A-R-R-G-L
K1P1.2	K-T-I-A-S-D-E-E-A-R-R-G-L
K1P2	K-P-S-V-Q-I-Q-V-Y-Q-G-E-R-E-I-A-S-H
K1P3	K-L-Q-A-A-P-A-L-D-R-L
K1P4	K-G-G-R-G-I-L-G-F-V-F-T-L
K1P5	K-A-M-R-M-A-T-P-L-L-M-Q-A
K1P1P4	K-T-I-A-Y-D-E-E-A-R-R-G-G-R-G-I-L-G-F-V-F-T-L
M2K1P1	M-K(M)-T-I-A-Y-D-E-E-A-R-R-G
M3K2P1	M-K(M)-K(M)-T-I-A-Y-D-E-E-A-R-R-G
M4K3P1	M-K(M)-K(M)-K(M)-T-I-A-Y-D-E-E-A-R-R-G
M5K4P1	M-K(M)-K(M)-K(M)-K(M)-T-I-A-Y-D-E-E-A-R-R-G
M6K5P1	M-K(M)-K(M)-K(M)-K(M)-K(M)-T-I-A-Y-D-E-E-A-R-R-G
M2K1P1.1	M-K(M)-T-I-Q-Y-D-E-E-A-R-R-G-L
M2K1P1.2	M-K(M)-T-I-A-S-D-E-E-A-R-R-G-L
M2K1P2	M-K(M)-P-S-V-Q-I-Q-V-Y-Q-G-E-R-E-I-A-S-H
M2K1P3	M-K(M)-L-Q-A-A-P-A-L-D-R-L
M2K1P4	M-K(M)-G-G-R-G-I-L-G-F-V-F-T-L
M2K1P5	M-K(M)-A-M-R-M-A-T-P-L-L-M-Q-A
M2K1P1P4	M-K(M)-T-I-A-Y-D-E-E-A-R-R-G-G-R-G-I-L-G-F-V-F-T-L

Mass spec data of the peptides:

	Formula	MH ⁺ calc(av)	MH ⁺ meas(av)
K1P1	C ₅₉ H ₉₇ N ₁₉ O ₂₁	1409.5	1409.1
K2P1	C ₆₅ H ₁₀₉ N ₂₁ O ₂₂	1537.7	1536.9
K3P1	C ₇₁ H ₁₂₁ N ₂₃ O ₂₃	1665.9	1666.2
K4P1	C ₇₇ H ₁₃₃ N ₂₅ O ₂₄	1794.1	1793.6
K5P1	C ₈₃ H ₁₄₅ N ₂₇ O ₂₅	1922.2	1922.2
K1P1.1	C ₆₇ H ₁₁₁ N ₂₁ O ₂₃	1579.7	1579.4
K1P1.2	C ₅₉ H ₁₀₄ N ₂₀ O ₂₂	1446.6	1446.9
K1P2	C ₉₀ H ₁₄₅ N ₂₇ O ₂₉	2070.3	2068.5
K1P3	C ₅₃ H ₉₄ N ₁₆ O ₁₅	1196.4	1196.6
K1P4	C ₈₅ H ₁₀₅ N ₁₇ O ₁₅	1365.6	n.d.
K1P5	C ₆₂ H ₁₁₂ N ₁₈ O ₁₆ S ₃	1462.9	1464.0
K1P1P4	C ₁₁₆ H ₁₈₅ N ₃₃ O ₃₃	2571.0	2570.0
M2K1P1	C ₈₅ H ₁₂₇ N ₂₁ O ₃₃ S ₂	2036.2	2035.3
M3K2P1	C ₁₀₄ H ₁₅₄ N ₂₄ O ₄₀ S ₃	2477.7	2476.5
M4K3P1	C ₁₂₃ H ₁₈₁ N ₂₇ O ₄₇ S ₄	2919.2	2917.2
M5K4P1	C ₁₄₂ H ₂₀₈ N ₃₀ O ₅₄ S ₅	3360.7	3360.9
M6K5P1	C ₁₆₁ H ₂₃₅ N ₃₃ O ₆₁ S ₆	3802.2	3796.7
M2K1P1.1	C ₉₃ H ₁₄₁ N ₂₃ O ₃₅ S ₂	2206.4	2206.2
M2K1P1.2	C ₈₅ H ₁₃₄ N ₂₂ O ₃₄ S ₂	2073.2	2074.1
M2K1P2	C ₁₁₆ H ₁₇₅ N ₂₉ O ₄₁ S ₂	2696.9	2697.3
M2K1P3	C ₇₉ H ₁₂₄ N ₁₈ O ₂₇ S ₂	1823.0	1822.0
M2K1P4	C ₉₁ H ₁₃₅ N ₁₉ O ₂₇ S ₂	1992.3	1992.1
M2K1P5	C ₈₈ H ₁₄₂ N ₂₀ O ₂₈ S ₅	2089.5	2090.5
M2K1P1P4	C ₁₄₂ H ₂₁₅ N ₃₅ O ₄₅ S ₂	3197.6	3195.3

Dendritic cell culture

Dendritic cells were cultured according to the protocol described by Sallusto and Lanzavecchia. Routinely the cells 5 were cultured for 7 days. All dendritic cell cultures were checked for the expression of typical dendritic cell surface markers by fluorescence activated cell sorter analysis on day 7. The cell surface markers analysed included CD1a, HLA-class I and class II, CD4 and the mannose receptor. All cultures 10 were found to be more than 95% positive for these markers. Subsequently the cells were frozen in a mixture of RPMI 1640 (Gibco) supplemented with 20% serum and 10% DMSO and stored in liquid nitrogen until usage.

T CELL CLONES

15 The T cell clones have been described in detail elsewhere (Anderson et al, Geluk et al, Oftung et al). Clone RP15.1.1 is specific for the peptide corresponding to amino acid 3-13 of *Mycobacterium leprae* HSP65. This peptide is designated P1. The response to the peptide is HLA-DR3 20 restricted. The T cell clone R3F7 is specific for the peptide corresponding to amino acid 413-424 of the *Mycobacterium leprae* HSP70. This peptide is designated P2. The response to the peptide is HLA-DR2 restricted. The T cell clone R2F10 is specific for the peptide corresponding to amino acid 418-427 25 of the *Mycobacterium leprae* HSP60. This peptide is designated P3. The response to the peptide is HLA-DR2 restricted. The T cell clones were expanded by specific stimulation with the appropriate peptide in the presence of autologous 30 irradiated (3000 RAD) peripheral blood mononuclear cells as antigen presenting cells. Subsequently the cells were frozen in a mixture of RPMI 1640 supplemented with 20% serum and 10% DMSO and stored in liquid nitrogen until usage.

Proliferation assay

For the measurement of proliferation 10.000 cloned T cells were mixed with 10.000 irradiated cultured dendritic cells. To this mannosylated or non-mannosylated peptide was 5 added in a previously determined concentration range. The total volume of the culture was 200 µl. All cultures were carried out in triplicates. Culture was at 37°C in a 5% CO₂ incubator for 48 hours. Next 1 µCi [³H]-thymidine was added and the culture continued for another 16 hours. Subsequently 10 the cultures were harvested and the [³H]-thymidine incorporation was measured using a beta-plate counter (Pharmacia) using standard protocols.

RESULTS

We have investigated if the in vitro mannosylation of 15 immunogenic peptide enhances their immunogenicity. For this purpose synthetic variants of three known antigenic peptides were synthetized with the addition of an N-terminal lysine residue. The peptides used were (single amino acid code): K-T-I-A-Y-D-E-E-A-R-R-G (designated peptide KP1), K-P-S-V-Q-I- 20 Q-V-Y-Q-G-E-R-E-I-A-S-H (designated peptide KP2) and K-L-Q-A-A-P-A-L-D-R-L (designated peptide KP3). The P1 peptide binds specifically to HLA-DR3 and is recognized by the human T cell clone RP15.1.1. The P2 and P3 peptide are recognized by the human T cell clones R3F7 and R2F10 respectively when bound to 25 HLA-DR2. A portion of these peptides was bis-mannosylated by the covalent attachment of mannose to both available primary amines on the N-terminal lysine residue. The peptides are designated M-K(M)-T-I-A-Y-D-E-E-A-R-R-G (M2KP1), M-K(M)-P-S-V-Q-I-Q-V-Y-Q-G-E-R-E-I-A-S-H (M2KP2) and M-K(M)-L-Q-A-A-P-A- 30 L-D-R-L (M2KP3) where M stands for an covalently linked mannose moiety. Both the mannosylated and non-mannosylated forms of these peptides were purified by preparative reversed phase HPLC. The correct mannosylation was checked by mass

spectral analysis of the purified product. The exact protocol used is described herein.

Human monocyte-derived dendritic cells were cultured according to a standard protocol described above. The 5 monocytes were isolated from the peripheral blood of an HLA-DR3 (DR17, DRbeta1*0301) positive and an HLA-DR2 positive healthy individual. The dendritic cells were co-cultured with the appropriate peptide specific T cells clones in the presence or absence of a concentration range of both the 10 mannosylated and non-mannosylated versions of the peptides. Specific proliferation was measured by the incorporation of [3H]-thymidine after 48 hours of culture. A similar experiment was performed using peripheral blood mononuclear 15 cells as antigen presenting cells instead of dendritic cells. The exact protocol used is described herein.

In those experiments where dendritic cells were used as antigen presenting cells and the T cell clones R2F10 and RP15.1.1 as responding cells it was observed that for the induction of half maximal proliferation approximately 3,000-20 to 10,000-fold less of the mannosylated peptide was required compared to the non-mannosylated analog. In those experiments where dendritic cells were used as antigen presenting cells and the T cell clone R3F7 as responding cell it was observed that for the induction of half maximal proliferation 25 approximately 100- to 300-fold less of the mannosylated peptide was required compared to the non-mannosylated analog.

In those experiments where peripheral blood mononuclear cells were used as antigen presenting cells and the T cell clones R2F10 and RP151.1 as responding cells it was observed 30 that for the induction of half maximal proliferation approximately 1,000- to 3,000-fold less of the mannosylated peptide was required compared to the non-mannosylated analog. In those experiments where peripheral blood mononuclear cells were used as antigen presenting cells and the T cell clone 35 R3F7 as responding cell it was observed that for the induction of half maximal proliferation approximately 3- to

10-fold less of the mannosylated peptide was required compared to the non-mannosylated analog.

It is of importance to note that the clone R3F7 requires at least 10-fold less non-mannosylated peptide for the induction of half-maximal proliferation compared to the clones R2F10 and RP15.1.1 and that the diminished effect of mannosylation in the case of the clone R3F7 may relate to the higher sensitivity of this clone.

In all experiments in which dendritic cells were used as antigen presenting cells, clone RP15.1.1 as responder cell and M2KP1 peptide, half maximal proliferation was observed at a peptide concentration of approximately 1 femtomol/ml. For the other two clones this was approximately 10 to 30 femtomol/ml. In the culture system used this corresponds to the availability of approximately 10,000 to 300,000 bis-mannosylated peptides for each dendritic cell. Since the number of mannose binding sites on cultured human dendritic cells is estimated to be 1.7×10^6 (Avrameas, A., et al. (1996)) this indicates that an absolute shortage of the mannosylated peptide resulting in suboptimal occupancy of the mannose receptor on the dendritic cells directly correlates with diminished antigenic stimulation of the responding peptide specific T cells.

Next we tested the influence of multiple mannose residues on the P1 peptide on presentation by dendritic cells. For this purpose the P1 peptide was synthetized with the addition of 1, 2, 3, 4 and 5 lysine residues at the N-terminus (KP1, K2P1, K3P1, K4P1 and K5P1). These peptides were subsequently mannosylated and purified (M2KP1, M3K2P1, M4K3P1, M5K4P1 and M6K5P1) and tested for their capacity to induce proliferation of the T cell clone RP15.1.1 in the presence of DR3 positive cultured dendritic cells. Virtually identical dose response-curves were obtained with all these peptides, inducing half-maximal proliferation at a concentration of approximately 1 femtomol/ml. Thus, under these conditions the addition of more than two mannose

residues to the peptide appears not relevant for uptake and presentation by dendritic cells to T cells.

Next we tested if a short exposure of dendritic cells to a mannosylated peptide was sufficient for the induction of proliferation of the T cell clone RP15.1.1. Dendritic cells were incubated at 37°C with either P1 or M2KP1 peptide and either washed directly with excess ice cold medium (0 minute incubation time point) or after a 3 and 10 minute incubation period. Subsequently the washed dendritic cells were co-cultured with clone RP15.1.1 and the proliferation measured after 48 hours. At both the 3 and 10 minute time point both the mannosylated (M2KP1) and the non-mannosylated peptide (P1) are able to induce strong proliferation of the T cell clone. At the 0 min time point, however, only the mannosylated peptide (M2KP1) induces strong proliferation. Thus, binding of mannosylated peptide to the receptor is fast and only a short exposure of the dendritic cell to a mannosylated ligand is required for the internalisation and subsequent presentation of an immunogenic peptide.

We also examined the effect of mannosylation on the induction of recall T helper cell responses. For this purpose the proliferative response of peripheral blood mononuclear cells of an individual known to be a responder to the HSP65 3-14 peptide was determined, against the non-mannosylated and mannosylated HSP65 3-14 peptides. It was observed that for the induction of a proliferative response in these peripheral blood mononuclear cells 1000 - 10.000 fold less of the mannosylated peptide was required compared with its non-mannosylated analog. Thus, in addition to be superior in stimulating human T cell clones, the mannosylated peptides are also superior in stimulating resting T cells in peripheral blood. These results indicate that mannosylated compounds could be superior for vaccination purposes.

Finally we tested if mannosylation of peptides with antagonistic properties leads to more efficient induction of T cell non-responsiveness. Substitution analogs of peptide (K1)P1 have been described (Geluk et al., J. Immol. 149;2864,

1992) that can block the response to (K1)P1 itself. These substitution analogs therefore have antagonistic properties but could only block the response to the agonist when added in large excess (approximately 1000-fold). To test if
5 mannosylation of these antagonists would lead to more efficient blocking we synthesized bis-mannosylated forms of these antagonists. We observed that for the blocking of the T cell response to the agonist approximately 1000-fold less of the mannosylated antagonist was needed compared the non-mannosylated antagonist. An unrelated bis-mannosylated peptide (M2K1P5) did not block the response to the agonist.
10

LEGEND

Table 1 gives a list of peptides which are very suitable to be used according to the present invention.

- 5 This list is by no means exhaustive or limiting.

Figure 1. Mannosylation of peptide strongly enhances its presentation by mannose receptor positive cultured human dendritic cells.

10

Figure 2. Mannosylation of antagonist peptide strongly enhances its presentation by mannose receptor positive cultured dendritic cells.

ABBREVIATIONS

	ACH	α -cyano-4-hydroxycinnamic acid
	DC	Dendritic cell
5	DMA	N,N-dimethylacetamide
	DMSO	Dimethylsulfoxide
	Fmoc	9-fluorenylmethoxycarbonyl
	HSP	Heat shock protein
	NMM	N-methylmorpholine
10	NMP	N-methylpyrrolidone
	PyBop	Benzotriazole-1-yl-oxy-tris-pyrrolidino- phosphonium hexafluorophosphate
	TFA	Trifluoroacetic acid

Table 1

peptide	source	restriction element
VDDTQFVRFDSDAASQMEPR	HLA-A2	DR4
5 DLSSWTAADTAAQITQR	HLA-B62	DR4
DNPEYSPDPSIYAYD	Calreticulin	DR4

Table 1 continued

	peptide	source	restriction element
5	NIQQLTEQPQVTNGVQN TKISDFGSF ₁ GFK GFKGSEDLGEGLKAV XGGVSVGGGASQWGN TLRAGRVANQFDDASQAIN	OMP OMP OMP OMP OMP	Class 1 Class 1 Class 1 Class 1 Class 1
10	XGGDSNNDVASQLQIFK XGGF\ S/G\ F/S\ G XGGYYTKDTNNNL AVVGKPGSDVYYA Y/A\ F/K\ Y/ARHANVGRN	OMP OMP OMP OMP OMP	Class 1 Class 1 Class 1 Class 1 Class 1
15	XANVGRNAFELFLIGSATTSDEAKG DEAKGTDPLKNHQVHRLTGGY XGGLENGDKAKTKNSTTE VPRISYAHGFDLIERGKKKG XGGERGKKGENTSYDQ	OMP OMP OMP OMP OMP	Class 1 Class 1 Class 1 Class 1 Class 1
20	KRNTGIGNYTQINA A	OMP	Class 1
	NYKNGGFAGNYAFKYARNHANVG RNAFELFLIGS VHRLTGGYEEGGLNLALAAQLDLS		
25	TKISDFGFIGFKGSELDLGD IGLAGEFGTRLAGRVA NQFD YRGNAVPRISYAHGFDIE RISYAHGFDLIERGKKGENT	OMP OMP OMP OMP	
30	YRGNAVPRISYAHGFDIE NTGIGNYTQINAASVGLRHK	OMP OMP	

Table 1 continued

	peptide	source	restriction element
5	AYGLDFYIL	p15	A24
	QDLTMKYQIF	decapeptide	A2/A11
	MLLAVLYCL	tyrosinase	A2
10	YMDGTMSQV	tyrosinase	A2
	YMNGTMSQV	tyrosinase	
	AFLPWHLRLFL	tyrosinase	A24
	MLLAVLYCL	tyrosinase	A2
	YMNGTMSQV	tyrosinase	A2
15	YMDGTMSQV	tyrosinase	A2
	SEIWRDIDF	tyrosinase	B44
	YLEPGPVTA	gp 100/Pmel-17	A2
	LLDGTATLRL	gp 100/Pmel-17	A2
20	KTWGQYWQV	gp 100/Pmel-17	A2
	ITDQVPFSV	gp 100/Pmel-17	A2
	VLYRYGSFSV	gp 100/Pmel-17	A2
	KTWGQYWQV	154-162	gp 100/Pmel-17
	ITDQVPGSV	209-217	gp 100/Pmel-17
25	YLEPGPVTA	280-288	gp 100/Pmel-17
	LLDGTATLRL	457-466	gp 100/Pmel-17
	VLYRYGSFSV	476-485	gp 100/Pmel-17
	AAGIGILTV	Melan-A/Mart-1	A2
30	ILTIVLGV	Melan-A/Mart-1	A2
	EADPTGSHSY	Mage-1	A2
	SAYGEPRKL	Mage-1	Cw*1601
	EVDPIGHLY	Mage-3	A1
	FLWGPRALV	Mage-3	A2
35	AARAVFLAL	Bage	Cw*1601
	YRPRPRRY	Gage	Cw6
	SPSSNRIRNT	Rage	B7
	ACDPHSGHFV	CDK4 (R24 mutation)	A2
	EEKLIVVLF	MUM-1 (mutated intron)	B44

Table 1 continued

	peptide	source
5	CLLDILDTAGL	61-Leu p21ras
	CLLDILDTA	wild-type p21ras
	GILGFVFTL	Influenza matrix
	ALWGFFGVL	Unknown (xeno-)
	ALWGFFPVI	Idem
10	YLEPGPVTA	pMel-17 (gp100)
	AAGIGILTV	Mart-1/Melan-A
	AAGIGILTV	Mart-1/Melan-A
	EAAGIGILTV	Mart-1/Melan-A
15	GILTVALGV	Mart-1/Melan-A
	ALMDKSLHV	Mart-1/Melan-A
	GILGFVFTL	Flu-M1
	EAAGIGILTV	Melan-A
20	AAGIGILTV	Melan-A
	YLEPGPVTA	gp100
	LLDGTATLRL	gp100
	KTWGQYWQV	gp100
	YMNGTMSQV	Tyrosinase
25	MLLAVALYCL	Tyrosinase

Table 1 continued

peptide	source	restriction element
5 116-129	Dermatophagoides pterouyssinus p2	DR1
29-42	Dermatophagoides pterouyssinus p2	DR1 (3,4)

Table 1 continued.

	peptide	source	restriction elements
5	NP73-92	Influenza virus nucleoprot.	DR1
	NP242-262	Influenza virus nucleoprot.	DR1
	NP206-229	Influenza virus nucleoprot.	DR1
	NP297-318	Influenza virus nucleoprot.	DR1
	NP302-313	Influenza virus nucleoprot.	DR1
10	NP404-415	Influenza virus nucleoprot.	DR1
	NP147-167	Influenza virus nucleoprot.	DR2
	NP216-229	Influenza virus nucleoprot.	DR2
	NP401-419	Influenza virus nucleoprot.	DR2
	NP260-283	Influenza virus nucleoprot.	DR7
15	NP335-342	Influenza virus nucleoprot.	DQw5
	NP284-391	Influenza virus nucleoprot.	B27
	NP338-344	Influenza virus nucleoprot.	B37
	NP338-345	Influenza virus nucleoprot.	B37
	NP417-428	Influenza virus nucleoprot.	B35
20	SAAFEDLRVLSFIKG	Influenza A virus nucleopr.	B37
	FEDLRVLS	Influenza A virus nucleopr.	B37

Table 1 continued

(Rammensee et al)

5	peptide	source	restriction elements
	EADPTGHSY	Mage-1 161-169	A1
	VSDGGPNLY	Influenza A PBI 591-599	A1
	CTELKLSDY	Influenza A NP 44-52	A1
10	EVDPIGHLY	MAGE-3	A1
	ILKEPVHGV	HIV-1 RT 476-484	A*0201
	ILGFVFTLTV	Influenza matrix protein 59-68	A*0201
	LLFGYPVYV	HTLV-1 tax 11-19	A*0201
15	GLSPTVWLSV	Hepatitis B sAg 348-357	A*0201
	WLSLLVPFV	Hepatitis B sAg 335-343	A*0201
	FLPSDFFPSV	Hepatitis B Nucleocapsid 18-27	A*0201
	CLGGLLTMV	EBV LMP2 426-434	A*0201
	FIAGNSAYEYV	HCMV glycoprotein B 618-628	A*0201
20	KLGEFYNQMM	Influenza B NP 85-94	A*0201
	KLVALGINAV	HCV-1 1406-1415	A*0201
	DLMGYIPLV	HCV core 132-140	A*0201
	RLVTLKDIV	HPV 11 E7 4-12	A*0201
	MLLALLYCL	Tyrosinase 1-9	A*0201
25	AAGIGILTV	Melan-A/Mart 1	A*0201
	YLEPGPVTA	pmel 17/gp100	A*0201
	ILDGTATLRL	pmel 17/gp100	A*0201
	RLRDILLIVTR	HIV-1 env gp41 768-778	A*0205
30	QVPLRPMTYK	HIV-1 nef 73-82	A*0205
	TVYYGVPVWK	HIV-1 env gp120 36-45	A*0205
	RLRPGGKKKK	HIV-1 gag p17 20-29	A*0205
	IIRGSVAHK	Influenza NP 265-273	A*0205
35	AVILPPLSPYFK	HSB 66 EST 18-29	A*1101
	ASFDKAKLKK	Thymosin β -10 11-20	A*1101
	IVTDFSVIK	EBNA 4 416-424	A*1101
	RYLKDDQQLL	HIV gp 41 583-591	A24

Table 1 continued

	peptide	source	restriction element
5	STLPETTVVRR	Hepatitis BcAg 141-151	A*3101
	IVGLNKIVR	HIV 224 gaga 267-275	A*3302
10	STLPETTVVRR	Hepatitis B cAg 141-151	A68.1
	TPGPGVRYPL	HIV-1 nef 128-137	B7
	ELRSRYWAI	Influenza NP 380-388	B8
15	FLRGRAYGL	EBNA 3 339-347	B8
	EIYKRWIIL	HIV gagP24 262-270	B8
	GEIYKRWII	HIV gagP24 261-269	B8
	EIKDTKEAL	HIV gagP24 93-101	B8
20	SRYWAIRTR	Influenza NP 383-391	B*2705
	RRRWRRRLTV	EBNA LMP2 236-244	B*2705
	RRYPDAVYL	Measles F protein 438-446	B*2705
	RRIYDLIEL	EBNA 3C 258-266	B*2705
	KRWIILGLNK	HIV-1 gag p24 265-274	B*2705
25	GRAFVTIGNK	HIV-1 gp120 314-322	B*2705
	RRKAMFEDI	HSP 60 284-292	B*2705
	KPKDELDY	P.falciparum CSP 368-375	B*3501
	KSKDELDY	P.falciparum CSP 368-375	B*3501
30	KPNDKSLY	P.falciparum LS 1850-1857	B*3501
	ASRCWVAM	HCV E1 235-242	B*3501
	EDLRVLSFI	Influenza NP 339-347	B*3701
35	EENLLDFVRF	EBNA 6 130-139	B*4403
	ILGNKIVRMY	HIV gag 267-276	B62
	QMVHQAISPRTL	HIV gag 141-152	Cw*0301

Table 1 continued

SFNCGGEFF	HIV-1 gp 120 380-388	Cw*0401
PV VHFFKNIVT	MBP 98-95	DRB1*1501
5 PK YVKQNTLKL AT	HA 307-319	DRB1*1501
LQAAPALDKL	HSP65 418-427	DRB5*0101
VHF FKNIVTPRT P	MBP 87-99	DRB5*0101
ASD YKSAHKGFK GVD	MBP 131-145	DRB5*0101
KG FKGVDAQGT LSKI	MBP 139-153	DRB5*0101
10 TGHGARTSTEPTTDY	EBV gp220 592-606	DR17/DRw52
KELKRQYEKKLRQ	EBV tegument p140 1395-1407	DR17/DRw52

Table 1 continued

	(Hammer et al)	source	restriction element
	peptide		
5	AKFYRDPTAFGSG	Link 61-73	DR4
	YNLFNFHEAQQAAL	Link 170-182	DR4
	DVFAFTSNFNGRF	Link 249-261	DR4
	TKLTYDEAVQAAL	Link 268-280	DR4
10	LTLVAAVLRAQG	Collagen 13-25	DR4
	DPLQYMRADQAAG	Collagen 1165-1177	DR4
	QYMRADQAAGGLR	Collagen 1168-1180	DR4
	MTFLRLLSTEGSQ	Collagen 1306-1318	DR4
	TFLRLLSTEGSQN	Collagen 1307-1319	DR4
15	KALLIQGSNDVEI	Collagen 1340-1352	DR4
	VEIRAEGNRSRFTY	Collagen 1350-1362	DR4
	FGLQLELTTEGMRF	hsp65 181-193	DR4
	EGMRFDKGYISGY	hsp65 189-201	DR4
	PYILLVSSKVSTV	hsp65 216-228	DR4
20	KAMLQDMAILTGG	hsp65 284-296	DR4
	GLFLTTEAVVADK	hsp65 509-521	DR4
	YILFAHLVNVKVHI	Fillagrin 9-21	DR4
	FAIINLFNEYSKK	Fillagrin 34-46	DR4
	GSFLYQVSTHEQS	Fillagrin 491-503	DR4
25	SFLYQVSTHEQSE	Fillagrin 492-504	DR4
	SSFSQDRDSQAQS	Fillagrin 644-656	DR4
	GSFLYQVSTHEQS	Fillagrin 815-827	DR4
	SFLYQVSTHEQSE	Fillagrin 816-828	DR4
	GSFIYQVSTHEQS	Fillagrin 1139-1151	DR4
30	SFIYQVSTHEQSS	Fillagrin 1140-1152	DR4

Table 1 continued

(Vogt et al) peptide	source	restriction element
5 PKYVKQNTLKLAT	HA (307-319)	DRB5*0101
LQAAPALDKL	HSP65 (418-427)	DRB5*0101
PVVHFFKNIVTPRTPPPSQGK	MBP (85-105)	DRB5*0101
ASDYKSAHKGFKGVD	MBP (131-154)	DRB5*0101
10 KGFKGVDAQGTLSKI	MBP (139-153)	DRB5*0101
PVVHFFKNIVTPRTPPPSQGK	MBP (82-105)	DRB5*0101
GTLSKIFKLGGRDSRSG	MBP (148-162)	DRB5*0101

Table 1 continued

	(Chicz et al) peptide	source	restriction elements
5			
	ATSTKKLHKEPATLIKAIDG	NASE 1-20	DR1
	PATLIKAIDGDTVKLMLYKGQ	NASE 11-30	DR1
	DRVKLMYLGQPMTGFLVD	NASE 21-40	DR1
	VAYVYKPNNTHEQHLRRKSEA	NASE 111-130	DR1
10	QKQEPIDKELYPLTLSL	HIV p13 97-112	DR1
	GARASVLSGGGLDKWE	HIV p17 1-16	DR1
	RTLYQNVGTYVSVGTSTLNK	Influenza HA 187-206	DR1
	PKYVKQNTLKLAT	Influenza HA 307-319	DR1
	LKKLVFGYRKPLNDI	P.falciparum p190 249-263	DR1
15	KHIEQYLKKIKNS	P.falciparum CS 329-341	DR1
	GRTQDENPVVHFFKNIVTPRTPPP	Myelin basic protein 75-98	DR1
	PLKAEIAQRLEDV	Influenza matrix 19-31	DR1
	RQILGQLQPSLQTGSE	HIV p17 57-72	DR1
	INTKCYKLEHPVTGCG	PLA ₂ 85-100	DR1
20	YKLNFYFDLLRAKL	P.falciparum P190 211-224	DR1
	ERFAVNPGLLERSEGC	HIV p17 41-56	DR1
	EALVRQLAKVAYVYKPNNT	NASE 101-120	DR1
	PIVQNLQGQMVMHQAIS	HIV p25 1-16	DR1
	KMYFNLINTKCYKLEH	PLA ₂ 79-94	DR1

Table 1 continued

(Geluk et al)	
peptide	source
5	
LPKPPKPVSKMRMATPLLMQALPM	Li p80-103
PKYVKQNTLKLAT	HA p308-319
KTIAYDEEARR	hsp65 p3-13

Table 1 continued

(Wang et al)	peptide	source	restriction elements
5	MSLQRQFLR	gp75	
(Gouilloux et al)			
10	VLPDVFIRC	N-acetylglucosaminyltransferase	A2

Table 1 continued

	peptide	source	restriction elements
5	KLPDLCTEL	HPV 18 E6 (13-21)	A2.1
	SLQDIEITC	HPV 18 E6 (24-32)	A2.1
	LQDIEITCV	HPV 18 E6 (25-33)	A2.1
	EITCVYCKT	HPV 18 E6 (29-37)	A2.1
	KTVLELTEV	HPV 18 E6 (36-44)	A2.1
10	ELTEVFEFA	HPV 18 E6 (40-48)	A2.1
	FAFKDLFVV	HPV 18 E6 (47-55)	A2.1
	DTLEKLTNT	HPV 18 E6 (88-96)	A2.1
	LTNTGLYNL	HPV 18 E6 (93-101)	A2.1
	TLQDIVLHL	HPV 18 E7 (7-15)	A2.1
15	FQQLFLNLT	HPV 18 E7 (86-94)	A2.1
	QLFLNLTLSF	HPV 18 E7 (88-96)	A2.1
	LFLNLTLSFV	HPV 18 E7 (89-97)	A2.1
	LSFVCPWCA	HPV 18 E7 (97-102)	A2.1
20	YRDGNPYAV	HPV 16 E6 (61-69)	A1
	WTGRCMSCC	HPV 16 E6 (139-147)	A1
	MSCCRSSRT	HPV 16 E6 (144-152)	A1
	TTDLYCYEQ	HPV 16 E7(19-27)	A1
	EIDGPAGQA	HPV 16 E7 (37-45)	A1
25	HVDIRTLED	HPV 16 E7 (73-81)	A1
	AMFQDPQER	HPV 16 E6 (7-15)	A2.1
	KLPQLCTEL	HPV 16 E6 (18-26)	A2.1
	QLCTELQTT	HPV 16 E6 (21-29)	A2.1
30	LCTELQTTI	HPV 16 E6 (22-30)	A2.1
	ELQTTIHDI	HPV 16 E6 (25-33)	A2.1
	LQTTIHDI	HPV 16 E6 (26-34)	A2.1
	TIHDIILEC	HPV 16 E6 (29-37)	A2.1
	IHDIIECV	HPV 16 E6 (30-38)	A2.1
35	CVYCKQQLL	HPV 16 E6 (37-45)	A2.1
	FAFRDLCIV	HPV 16 E6 (52-60)	A2.1
	KISEYRHYC	HPV 16 E6 (79-87)	A2.1
	PLCDLLIRC	HPV 16 E6 (102-110)	A2.1
	TLHEYMLDL	HPV 16 E7(7-15)	A2.1

Table 1 continued

	YMLDLQPET	HPV 16 E7 (11-19)	A2.1
	MLDLQPETT	HPV 16 E7 (12-20)	A2.1
5	RLCVQSTHVV	HPV 16 E7 (66-74)	A2.1
	TLEDLLMGT	HPV 16 E7 (78-86)	A2.1
	LLMGTLGIV	HPV 16 E7 (82-90)	A2.1
	GTLGIVCPI	HPV 16 E7 (85-93)	A2.1
	TLGIVCPIC	HPV 16 E7 (86-94)	A2.1
10	AMFQDPQER	HPV 16 E6 (7-15)	A3.2
	IILECVYCK	HPV 16 E6 (33-41)	A3.2
	CVYCKQQLL	HPV 16 E6 (37-45)	A3.2
	VYCKQQLLR	HPV 16 E6 (38-46)	A3.2
15	QQLLRREVY	HPV 16 E6 (42-50)	A3.2
	IVYRDGNPY	HPV 16 E6 (59-67)	A3.2
	YAVCDKCLK	HPV 16 E6 (67-75)	A3.2
	AVCDKCLKF	HPV 16 E6 (68-76)	A3.2
	VCDKCLKFY	HPV 16 E6 (69-77)	A3.2
20	KFYSKISEY	HPV 16 E6 (77-83)	A3.2
	KISEYRHYC	HPV 16 E6 (79-87)	A3.2
	ISEYRHHCY	HPV 16 E6 (80-88)	A3.2
	RHYCYSLYG	HPV 16 E6 (84-92)	A3.2
	SLYGTTLEQ	HPV 16 E6 (89-97)	A3.2
25	TTLEQQYNK	HPV 16 E6 (93-101)	A3.2
	QQYNKPLCD	HPV 16 E6 (97-105)	A3.2
	LIRCINCQK	HPV 16 E6 (107-115)	A3.2
	HLDKKQRFH	HPV 16 E6 (125-133)	A3.2
	CMSCCRSSL	HPV 16 E6 (143-151)	A3.2
30	SCCRSSRTR	HPV 16 E6 (145-153)	A3.2
	CCRSSRTRR	HPV 16 E6 (146-154)	A3.2
	HYNIVTFCC	HPV 16 E7 (51-59)	A3.2
	YNIVTFCC	HPV 16 E7 (52-60)	A3.2
	CCKCDSTLR	HPV 16 E7 (58-66)	A3.2
35	KCDSTLRLC	HPV 16 E7 (60-68)	A3.2
	AMFQDPQER	HPV 16 E6 (7-15)	A11.2
	IILECVYCK	HPV 16 E6 (33-41)	A11.2
	CVYCKQQLL	HPV 16 E6 (37-45)	A11.2

Table 1 continued

	VYCKQQLLR	HPV 16 E6 (38-46)	A11.2
	QQLLRREVY	HPV 16 E6 (4-50)	A11.2
5	IVYRDGNPY	HPV 16 E6 (59-67)	A11.2
	YAVCDKCLK	HPV 16 E6 (67-75)	A11.2
	AVCDKCLKF	HPV 16 E6 (68-76)	A11.2
	VCDKCLFKY	HPV 16 E6 (69-77)	A11.2
	KISEYRHYC	HPV 16 E6 (79-87)	A11.2
10	ISEYRHYCY	HPV 16 E6 (80-88)	A11.2
	LIRCINCQK	HPV 16 E6 (107-115)	A11.2
	TGRCMSCCR	HPV 16 E6 (140-148)	A11.2
	CMSCCRSSR	HPV 16 E6 (143-151)	A11.2
	SCCRSSRTR	HPV 16 E6 (145-153)	A11.2
15	HYNIVTFCC	HPV 16 E7 (51-59)	A11.2
	YNIVTFCCK	HPV 16 E7 (52-60)	A11.2
	CCKCDSTLR	HPV 16 E7 (58-66)	A11.2
	VCPICSQKP	HPV 16 E7 (90-98)	A11.2
20	MHQKRTAMF	HPV 16 E6 (1-9)	A24
	LQTTIHDII	HPV 16 E6 (26-34)	A24
	VYCKQQLLR	HPV 16 E6 (38-46)	A24
	LLRREVYDF	HPV 16 E6 (44-52)	A24
	VYDFAFRDL	HPV 16 E6 (49-57)	A24
25	PYAVCDKCL	HPV 16 E6 (66-74)	A24
	KCLKFYSKI	HPV 16 E6 (72-80)	A24
	EYRHYCYSL	HPV 16 E6 (82-90)	A24
	HYCYSLYGT	HPV 16 E6 (85-93)	A24
	CYSLYGTTL	HPV 16 E6 (87-95)	A24
30	RFHNIRGRW	HPV 16 E6 (131-139)	A24
	RAHYNIVTF	HPV 16 E7 (49-57)	A24

REFERENCES

- Coste, J., Dufour, M.-N., Nguyen, D. and Castro, B. (1990) BOP and congenes: present status and new developments. In: J.E. Rivier and G.R. Marshall (Eds.) Peptides: Chemistry, Structure and Biology, Escom, Leiden, p. 885.
- 5 Fields, G.B. and Noble, R.L. (1990) Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. Int. J. Peptide Protein Res. 35, 161.
- Gausepohl, H., Kraft, M., Boulin, C. and Frank, R.W. (1990^a) A multiple reaction system for automated simultaneous peptide synthesis. In: E. Giralt and D. Andreu (Eds.) Peptides 1990, Escom, Leiden, p. 206.
- 10 Gausepohl, H., Kraft, M., Boulin, Ch. and Frank, R.W. (1990^b) Automated multiple peptide synthesis with BOP activation. In: J.E. Rivier and G.R. Marshall (Eds.), Peptides: Chemistry, Structure and Biology, Escom, Leiden, p.1003.
- 15 Rapp, W., Zhang, L. and Bayer, E. (1990) Continuous flow peptide synthesis on PSPOE-graft copolymers. In: R. Epton (Ed.) Innovation and Perspectives in Solid Phase Peptide Synthesis, SPCC (UK) Ltd, Birmingham, p. 205.
- 20 Sheppard, R.C. and Williams, B.J. (1982) Acid-labile resin linkage agents for use in solid phase peptide synthesis. Int. J. Peptide Protein Res. 20, 451.
- Anderson, D.C., Van Schooten, W.C.A., Janson, A., Barry, M.E., and De Vries, R.R.P. (1990). Molecular mapping of interactions between a *Mycobacterium leprae*-specific T cell epitope, the restricting HLA-DR2 molecule, and two specific T cell receptors. J. Immunol. 144, 2459-2464
- 25 Geluk, A., Van Meijgaarden, K.E., Janson, A.A.M., Drijfhout, J.W., Meloen, R.H., De Vries, R.R.P., and Ottenhoff, T.H.M. (1992). Functional analysis of DR17(3)-restricted mycobacterial T cell epitopes reveals DR17 binding motif and enables the design of allele specific competitor peptides. J. Immunol 149, 2864-2871

Offung, F., Geluk, A., Lundin, K.E.A., Meloen, R.H., Thole,
J.E.R., Mustafa, A.S., and Ottenhoff, T.H.M. (1994). Mapping
of multiple HLA class II-restricted T-cell epitopes of the
mycobacterial 70-kilodalton heat shock protein. *Infection and*

5 *Immunity* 62, 5411-5418

Sallusto, F. and Lanzavecchia, A. (1994). Efficient
presentation of soluble antigen by cultured human dendritic
cells is maintained by granulocyte/macrophage colony-
stimulating factor plus interleukin 4 and downregulated by

10 tumor necrosis factor α . *J. Exp. Med.* 179, 1109-1116

Avrameas, A., McIlroy, D., Hosmalin, A., Autran, B., Debré,
P., Monsigny, M., Roche, A.C., and Midoux, P. (1996).

Expression of a mannose/fucose membrane lectin on human
dendritic cells. *Eur. J. Immunol.* 26, 394-400

CLAIMS

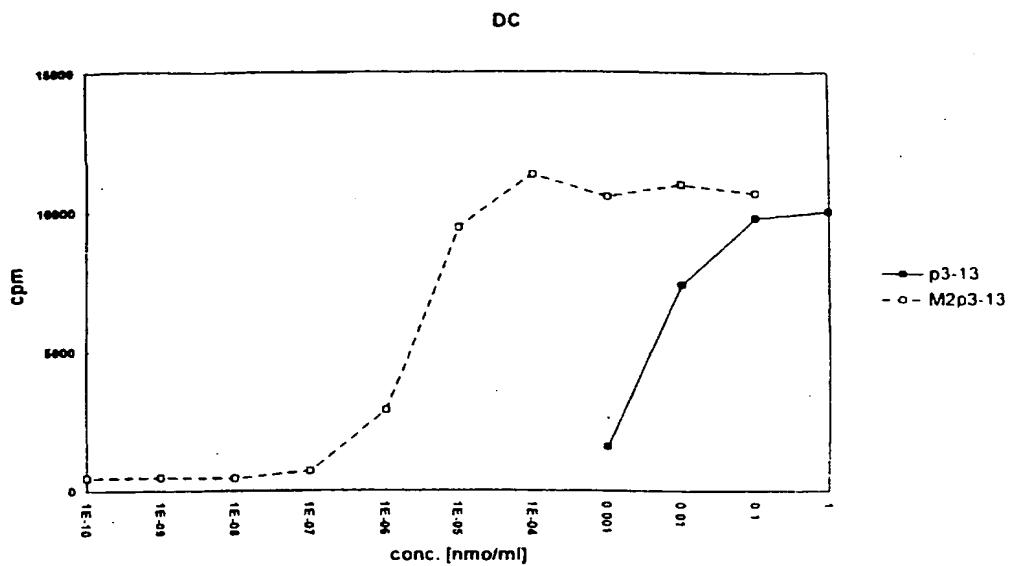
1. A method for enhancing uptake of a proteinaceous antigen or a peptide derived therefrom by antigen presenting cells having a mannose or similar receptor, whereby the antigen or peptide is provided with at least one and preferably two mannose groups or a functional equivalent thereof.
5
2. A method for enhancing antigen presentation by an antigen presenting cell comprising contacting said cell with a soluble antigen which has been provided with at least one and preferably two mannose groups or a functional equivalent thereof and allowing the cell to internalize and process said mannosylated antigen.
10
3. A method for producing a cell presenting a peptide antigen, whereby said peptide antigen is provided with at least one and preferably two mannose groups or a functional equivalent thereof, comprising contacting said cell with a soluble antigen which has been provided with a mannose group or a functional equivalent thereof and allowing the cell to internalize and process said mannosylated antigen.
15
4. A method according to claims 1-3 whereby the antigen presenting cell is a cell which presents peptides in the context of MHC-class II and/or MHC-class I.
20
5. A method according to claims 1-4 whereby the antigen presenting cell is a dendritic cell.
6. An antigen presenting cell obtainable by a method
25 according to claim 3.
7. A cell according to claim 6 which is a dendritic cell.
8. A method for improving the T cell response to a peptide antigen comprising contacting said T cell with a cell according to claim 6 or 7.
- 30 9. A vaccine preparation for the treatment or prophylaxis of infection by a pathogen comprising at least one proteinaceous antigen derived from said pathogen, which proteinaceous antigen is provided with at least one and

preferably two mannose groups or a functional equivalent thereof.

10. A vaccine preparation for the treatment or prophylaxis of infection by a pathogen comprising at least one peptide derived from said pathogen which peptide is provided with at least one and preferably two mannose groups or a functional equivalent thereof.

11. A peptide provided with at least one and preferably two mannose groups or a functional equivalent thereof for use in the preparation of a vaccine.

12. A proteinaceous antigen provided with at least one and preferably two mannose groups or a functional equivalent thereof for use in the preparation of a vaccine.



INTERNATIONAL SEARCH REPORT

International Application No.

PCT/NL 97/00536

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/35 A61K39/04

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>A. LANZAVECCHIA: "Mechanisms of uptake for presentation" CURRENT OPINION IN IMMUNOLOGY, vol. 8, no. 3, June 1996, LONDON GB, pages 348-354. XP002028910 see page 349, left-hand column, paragraph 3 see page 349, left-hand column, paragraph 5 - right-hand column, paragraph 1 --- -/-</p>	1-9

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 January 1998	12/02/1998
Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040 Tx. 31 651 epo nl. Fax: (+31-70) 340-3016	Fuhr, C

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 97/00536

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	F. SALLUSTO ET AL.: "Dendritic Cells Use Macropinocytosis and the Mannose Receptor to Concentrate Macromolecules in the Major Histocompatibility Complex Class II Compartment: Downregulation by Cytokines and Bacterial Products" JOURNAL OF EXPERIMENTAL MEDICINE, vol. 182, no. 2, August 1995, pages 389-400, XP000647847 see page 396, right-hand column, paragraph 3 - page 399, right-hand column, paragraph 2 ---	1-11
A	R.M. STEINMAN AND J. SWANSON: "The Endocytic Activity of Dendritic cells" JOURNAL OF EXPERIMENTAL MEDICINE, vol. 182, no. 2, 1 August 1995, pages 283-288, XP000647848 see page 284, right-hand column, last paragraph - page 285, left-hand column, paragraph 1 ---	1-11
A	EP 0 659 768 A (AUSTIN RESEARCH INST) 28 June 1995 see claims; examples ---	1-11
P,X	F. KONING: "Enhancement of HLA class II restricted antigen presentation by mannose-receptor-mediated uptake" BIOCHEMICAL SOCIETY TRANSACTIONS, vol. 25, no. 2, May 1997, pages 664-665, XP002053241 see the whole document -----	1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.

PCT/NL 97/00536

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0659768 A	28-06-95	AU 1308195 A AU 8172894 A WO 9518145 A CA 2135833 A JP 7206707 A	17-07-95 29-06-95 06-07-95 25-06-95 08-08-95